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REVIEW

New techniques, applications and perspectives in neuropeptide research

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ABSTRACT

Neuropeptides are one of the most diverse classes of signaling molecules and have attracted great interest over the years owing to their roles in regulation of a wide range of physiological processes. However, there are unique challenges associated with neuropeptide studies stemming from the highly variable molecular sizes of the peptides, low *in vivo* concentrations, high degree of structural diversity and large number of isoforms. As a result, much effort has been focused on developing new techniques for studying neuropeptides, as well as novel applications directed towards learning more about these endogenous peptides. The areas of importance for neuropeptide studies include structure, localization within tissues, interaction with their receptors, including ion channels, and physiological function. Here, we discuss these aspects and the associated techniques, focusing on technologies that have demonstrated potential in advancing the field in recent years. Most identification and structural information has been gained by mass spectrometry, either alone or with confirmations from other techniques, such as nuclear magnetic resonance spectroscopy and other spectroscopic tools. While mass spectrometry and bioinformatic tools have proven to be the most powerful for large-scale analyses, they still rely heavily on complementary methods for confirmation. Localization within tissues, for example, can be probed by mass spectrometry imaging, immunohistochemistry and radioimmunoassays. Functional information has been gained primarily from behavioral studies coupled with tissue-specific assays, electrophysiology, mass spectrometry and optogenetic tools. Concerning the receptors for neuropeptides, the discovery of ion channels that are directly gated by neuropeptides opens up the possibility of developing a new generation of tools for neuroscience, which could be used to monitor neuropeptide release or to specifically change the membrane potential of neurons. It is expected that future neuropeptide research will involve the integration of complementary bioanalytical technologies and functional assays.

KEY WORDS: Neuropeptides, Mass spectrometry, Peptide-gated ion channel, FaNaC/HyNaC, Immunochemistry, Electrophysiology

Introduction

Neuropeptides are a class of endogenous peptides that act as long-lasting neurotransmitters in the nervous system and other target organs. By signaling via synapses or volume transmission via diffusion, as well as via long-range signaling as circulating hormones, neuropeptides and their receptors play an important role in several key processes. When a neuron releases neuropeptides, the binding of the neuropeptide to its receptor on a receiving cell causes conformational changes within the receptor that, depending on the type of receptor, either open ion channels or activate coupled G proteins that can cause a series of downstream effects within the cell (van den Pol, 2012). As neuropeptides are a highly diverse class of signaling molecules in the brain and other peripheral organs, their structures, functions and localization are of great interest and relevance (Hughes and Woodruff, 1992). The extent of their implied roles in normal biological processes has been a point of focus in studies over the years (Hook and Bandeira, 2015; Zhang et al., 2014). Abnormalities in their expression can contribute to various neurological diseases by altering the function of specific neurons, and so understanding the mechanisms of neuropeptide signaling can help researchers to better understand these diseases and develop more focused and effective treatments (Beal and Martin, 2016). Furthermore, neuropeptides have been implicated in the regulation of normal biological functions, such as feeding regulation (Chen et al., 2010b; Gomes et al., 2013), and the adaptation to external factors, such as temperature fluctuation (Chen et al., 2014), and internal stress factors, including depression, anxiety and post-traumatic stress disorder (Kormos and Gaszner, 2013; Reichmann and Holzer, 2016). As a result, understanding the specific role individual neuropeptides play in response to interactions with the environment and in the execution of biological functions can provide a greater understanding of the underlying mechanisms at the cellular and systemic level. Investigations of the relationship between neuropeptides and their receptors are useful for the development of drug molecules for treating diseases or for otherwise manipulating interactions between peptidergic neurons, such as the treatment of specific symptoms (e.g. chemotherapy-induced emesis) (Hökfelt et al., 2003).

While neuropeptides are interesting biomolecules that have important roles in regulating a wide range of physiological processes, they have numerous characteristics that make them challenging to study. Because neuropeptides can be used as modulators for signaling locally between neurons as well as functioning as hormones that can travel a long distance to target sites, the *in vivo* concentrations can vary dramatically. Furthermore, low concentrations of neuropeptides can have profound effects – these signaling molecules are typically present at low endogenous concentrations, up to 1000-fold or lower than classical neurotransmitters and other metabolites (Romanova and Sweedler, 2015). This challenge is exacerbated by the lack of a

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Glossary

Depolarizing
Increasing the membrane potential (towards a less negative voltage)

Desensitizing
Becoming insensitive to a ligand upon prolonged exposure to it, causing, for example, an ion channel to close even though it has a ligand bound

Hyperpolarizing
Decreasing the membrane potential (towards a more negative voltage)

MS3 level
In mass spectrometry, during MS1 level acquisition, an *m*/*z* of interest is selected for fragmentation (tandem MS, MS/MS or MS2) to obtain key structural information. A MS2 ion can then be selected further to undergo another stage of fragmentation, which is referred to MS3 level.

Patch pipette
The recording electrode in a patch clamp electrode set up. The electrode is usually a glass micropipette.

Postsynaptic membrane
Synapses are the sites of contact of a neuron with another cell. At the presynaptic membrane, the neuron releases the transmitter. At the postsynaptic membrane, the other cell, which can for example be another neuron or a muscle cell, receives the transmitter. The presynaptic and postsynaptic cells are separated from each other by the synaptic cleft.

Sequence coverage
The percentage of a neuropeptide's sequence that is identified in a mass spectrometry experiment. Higher sequence coverage corresponds to a more confident identification for a given neuropeptide.

Western blotting
An antibody-based technique to visualize proteins in a sample. Classically, a separation, such as gel electrophoresis, is done prior to exposing the proteins to the antibodies.

digesting enzyme for a typical neuropeptide analysis workflow, as these molecules are products of proteolytic processing and post-translational modifications (PTMs) that occur inside cells or during transportation. As such, there is only one opportunity for detecting each peptide, unlike in 'bottom-up' proteomic studies, where a single unique tryptic fragment is sufficient to detect a protein (Fricker et al., 2006). This necessitates the development of highly sensitive detection methods in order to avoid large sample requirements. Additionally, sample processing methods need to be fast because, as with other signaling molecules, neuropeptides are prone to rapid degradation. Thus, it is often difficult to identify peptides as endogenous and not simply the product of a degraded larger protein, further complicating analysis (Schrader et al., 2014). Additionally, there is a large amount of variability between different neuropeptides, either owing to possession of different sequences but with the same mass or because of them having numerous PTMs (Li and Sweedler, 2008). Even when the structure has been identified, there are still complications. Neuropeptides can have the same structure but different functions or have different functions depending on the cell type and nearby receptors (Morimoto et al., 2008). Furthermore, as many isoforms exist for various neuropeptide families, localization of specific neuropeptides can be challenging owing to difficulties assigning mass spectral peaks to specific peptides (Hanrieder et al., 2012).

Despite these difficulties, much progress has been made over the years to characterize neuropeptides, including gaining information about their structure, function and localization within cells and the whole neuroendocrine system. Here, we focus on reviewing recent advancements made in developing techniques and applications to study neuropeptides and their receptors, while pausing to offer insights into the direction in which the field is moving. The areas described include structural elucidation of neuropeptides, methods for their localization, and their functional assessment, as depicted in Fig. 1 and summarized in Table 1, all of which are required to understand neuropeptide biology comprehensively. We also present a case study on the characterization of peptide-gated ion channels and how they might be modified into new tools for neuroscience. While space constraints mean that we do not intend to provide a comprehensive account of all recent publications, we nevertheless provide notable highlights of some key developments made within the past few years.

Elucidation of neuropeptide structures

Perhaps the most important information gained about neuropeptides relates to their structures (e.g. amino acid sequence, PTMs, folding pattern, binding sites), as these provide insights into their function and biological mechanisms. However, gaining this information can be challenging and cumbersome. While it has been almost a century since the first neuropeptide, substance P, was discovered (von Euler and Gaddum, 1931), and nearly 50 years since the sequence of that peptide was determined (Chang et al., 1971), technology has since developed impressively, and there are now records of almost 6000 neuropeptide sequences across all species (Wang et al., 2015).

Early work in structural elucidation relied on Edman degradation, a technique developed by Pehr Edman, in which peptides are reacted with phenyl isothiocyanate at the N-terminus and analyzed one amino acid at a time as each residue is removed (Edman, 1950). Successful sequencing using Edman degradation relies on the peptide being present in high concentrations (>1 picomolar) and at high purity. While Edman degradation is a classic method that allowed for the sequencing of many neuropeptides early on, it is less widely used, as other, more high-throughput, methods have emerged in recent years. The technique is still used in some applications, although mostly coupled with mass spectrometry (MS). For example, it has been successfully used to sequence a novel neuropeptide, Y-HS, in leeches (Liu et al., 2016c), to discover a novel arrangement of cysteine residues in a neuropeptide from a worm-hunting snail (Aguilar et al., 2013), and to determine the sequence of human and mouse urocortin 2, a member of the corticotropin-releasing factor neuropeptide family (Vaughan et al., 2013). Although Edman degradation has proven to be a useful addition to other techniques in these applications, the method has largely been replaced with higher-throughput and more-sensitive methods, such as MS, in the past decade.

Currently, MS serves as the method of choice for sequencing and determining the PTMs of neuropeptides (Gilsh and Vachet, 2003; Li and Sweedler, 2008; Potocnik et al., 2017; Romanova and Sweedler, 2015). MS has proven to be useful for detecting small amounts of peptides in complex biological samples, making it a high-throughput and versatile technique ideal for the study of endogenous neuropeptides. This advancement has enabled the emergence of 'neuropeptidomics', studying the entire neuropeptide complement as a whole either by comparing spectra to a database of known neuropeptides or *de novo* sequencing to discover new neuropeptides (Steen and Mann, 2004). Fig. 2 shows how MS spectra can be used to assign sequences by matching fragment ion masses to amino acids based on cleavage patterns and comparing the *de novo* sequences to those predicted based on genomic data.

Numerous studies have been performed using a variety of specific MS techniques. These techniques and their results have been comprehensively reviewed elsewhere (Buchberger et al., 2015;

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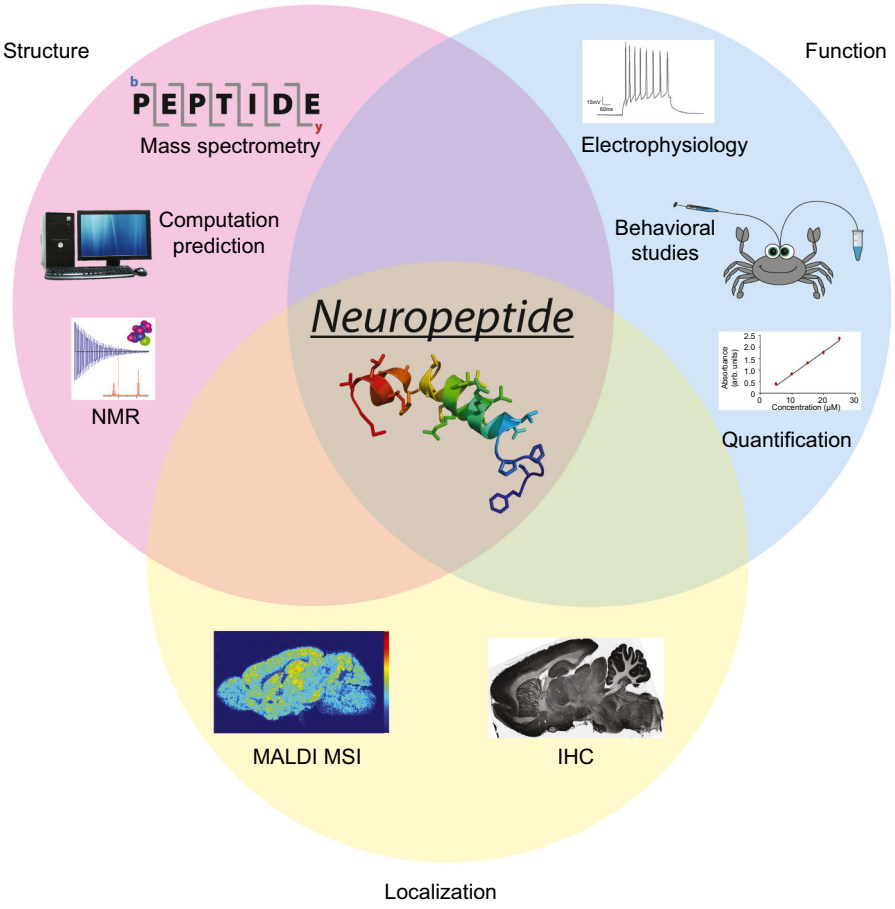

Fig. 1. A general depiction of the importance of structure, function and localization to provide key information about a neuropeptide. Several methods for each area of the Venn diagram are highlighted. For structure tools, MS, computational prediction and NMR are shown. MALDI MSI and IHC are the examples depicted for tools to provide localization. For understanding functionality, quantification, behavioral studies and electrophysiology are core techniques.

Romanova and Sweedler, 2015), but noteworthy strides have been made recently, such as developments in analyzing neuropeptides rich in disulfide bonds, which in the past has been a particularly laborious task. A new method has recently been developed for identifying disulfide bonds by alkylating peptides and then performing targeted fragmentation on disulfide-bonded peptides (Yu et al., 2015). Another method has integrated MS and nuclear magnetic resonance (NMR) techniques for rapid identification and characterization of disulfide bonds using only 4 ng of a peptide sample. The method involved studying the mass differences between folded and unfolded neuropeptides. As disulfide bond formation results in a mass difference of ~2 Da due to the loss of two hydrogen atoms, and these disulfide bonds are not present in unfolded peptides, the mass differences can be used to assess the number of disulfide bonds present in a folded peptide. The presence of disulfide bonds can then be confirmed with NMR (Anand et al., 2016). Another challenge with neuropeptidomics is that neuropeptides degrade substantially over time. A new, high-throughput framework for neuropeptide identification has recently been developed for fast high-throughput analysis, minimizing neuropeptide degradation. With this method, researchers were able to successfully identify thousands of neuropeptides and post-translational modifications (Secher et al., 2016). Additionally, neuropeptide sequence coverage has been shown to improve when coupling several techniques of fragmentation (e.g. high-energy collisional dissociation and electron-transfer dissociation) (Frese et al., 2013; Schmidlin et al., 2015; Shen et al., 2011), especially when utilizing dual fragmentation with electron-transfer high-energy collisional dissociation (EThcD) (Frese et al., 2012; Yu et al., 2017).

Advances have also been made in the characterization of novel neuropeptides from model organisms, expanding our knowledge of existing neuropeptides. By combining matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS to characterize the carpenter ant neuropeptidome, 39 neuropeptides were identified (Schmitt et al., 2015). The beetle neuropeptidome has also been expanded within the past year, with novel neuropeptides from the adipokinetic hormone family sequenced with ESI-MS and tandem MS. These were confirmed by co-eluting each naturally existing neuropeptide with its synthetic neuropeptide by means of liquid chromatography (LC) (Gäde et al., 2015, 2016). One other noteworthy group of organisms seeing significant growth in knowledge of its neuropeptidome has been members of the subphylum crustacea. Multidimensional MS techniques have been successfully implemented in both defining neuropeptidomes, such as that of the spiny lobster (Ye et al., 2015), and discovering numerous novel neuropeptides (Hui et al., 2013; Jia et al., 2013). As a complementary separation technique, ion mobility spectrometry (IMS) is currently experiencing rapid growth, and its use in conjunction with MS has allowed for a more comprehensive study of peptide structure (Kanu et al., 2008). Recently, ion mobility spectrometry (IMS) has been used for conformational studies investigating the role of the penultimate proline residue (Glover et al., 2015) and D-amino acid-containing peptide epimers (Jia et al., 2014; Pang et al., 2017), and it is expected that more structural knowledge will come from IMS in future studies.

The capabilities of MS for discovery and characterization of neuropeptides have been greatly assisted by the development of computational methods for predicting neuropeptides from precursor

Table 1. A summary of notable techniques commonly used to provide information about the three major areas of neuropeptide research: structure, localization and function

Area of interest	Technique	Description	Key references
Structure	Mass spectrometry	Determines sequences, PTMs and structural information	Secher et al., 2016; Gade et al., 2016; Glover et al., 2015
	In silico prediction	Predicts sequences and structure from precursor protein and gene sequences	Christie, 2014; Wong et al., 2016; Bigot et al., 2014
	NMR	Gives information into conformations and folding patterns	Robinson et al., 2016; Xie et al., 2015; Kaiser et al., 2015
	Spectroscopy	Uses characteristic peaks to identify folding patterns	Hegefeld et al., 2011; Schneider et al., 2016
	X-ray crystallography	Characterizes key structural sites with high spatial resolution	Hassler et al., 2014; Yin et al., 2014
Localization	Immuno assays	Enables localization for virtually any peptide using antibodies	Singh et al., 2016; Husson et al., 2009; Rowe and Elphick, 2012
	In situ hybridization	Target-specific expression mapping of neuropeptide-encoding genes	Levsky and Singer, 2003; Qian and Lloyd, 2003; Atkinson et al., 2016
	Promotor::reporter gene constructs	Enables transcript detection in living cells and organisms	Kim and Li, 2004; Clynen et al., 2010; Turek et al., 2016
	MSI	Capable of imaging entire neuropeptidomes without prior knowledge	Chen et al., 2016a; Mark et al., 2012; OuYang et al., 2015a
	Bio imaging and microscopy	Maps the architecture of the nervous system	Schmidt-Rhaesa et al., 2016; Fricker, 2012; Bixel et al., 2015
Function	Behavioral studies	Common first step to obtaining a general understanding of function to judging potential for disease treatment	Zhang et al., 2016; Kasica et al., 2016; Flores-Burgess et al., 2017
	Electrophysiology	Provides understanding of synaptic mechanisms	Kuksis and Ferguson, 2014; Li et al., 2016; Otopalik et al., 2017
	Quantitative analyses (western blotting, ELISA, MS, etc.)	Implies functions by measuring changes in neuropeptide levels due to specific behaviors or conditions	Liu et al., 2016a; Schmerberg and Li, 2013b; Bilgic et al., 2016

Each technique described has the potential to provide deep insight into neuropeptide biology, and often provide complementary information to other techniques. Several key references are indicated for each that demonstrate current trends in the field.

proteins and gene sequences. The area of *in silico* prediction has seen substantial growth in the past few years, with neuropeptidomes being predicted for a wide variety of organisms and novel neuropeptides being targeted for more focused analyses (Christie, 2014). Coupling these informatics approaches to other methods of analysis, particularly MS, has allowed for an expanded coverage of neuropeptidomes, which is important for species without a fully sequenced genome, especially when one transcript can produce several neuropeptides (Christie, 2014; Wong et al., 2016). For example, the crab *Cancer borealis* peptidome was doubled by mining its neural transcriptome (Christie and Pascual, 2016). As a complementary technique, researchers have also made use of *in silico* prediction methods to characterize neuropeptide receptors, which gives insight into both structural and functional properties by assessing similarities to previously characterized receptors (Bigot et al., 2014). Computational efforts have further been directed toward compiling databases of known neuropeptides to provide comprehensive coverage and compare neuropeptides detected in different species. The most recent of these, NeuroPep, contains almost 6000 entries (Wang et al., 2015). Resampling approaches are being developed to improve database matching, allowing for better identifications in terms of both quality and quantity (Akhtar et al., 2014).

By combining information about structure gained from MS with other powerful analytical tools, researchers have been able to gain better insight into the overall structural composition of neuropeptides. Various types of NMR techniques have been implemented for studying neuropeptides, which are particularly valuable for characterizing folding patterns. Fig. 3 shows an example of how several complementary NMR experiments can be combined to assess the structural conformation of a neuropeptide, as was done to determine the conformational patterns of the hormone pheromonotropin that controls larval sex pheromone production (Bhattacharya et al., 2015). Several recent advances have been made in understanding the secondary structure of neuropeptides. As an example, a precursor protein existing in marine snail venom was investigated by using solution NMR structural determination and was found to have a disulfide-directed β-hairpin fold, which initiates folding in other disulfide-containing areas of the peptide (Robinson et al., 2016). Some neuropeptides have been found to lack secondary structure, as in the case of an RFamide neuropeptide discovered in cone snail venom (Robinson et al., 2015). As structural characteristics are important for the interaction between neuropeptides and their receptors, many recent advances have used NMR to characterize these relationships, such as determining which conformations are important for biological activity, as has been investigated for various analogs of an allatostatin neuropeptide (Xie et al., 2015), as well as determining which part of the receptor neuropeptides were bound to, as has been accomplished with solid-state NMR for neuropeptide Y and its receptor (Kaiser et al., 2015). The relationship with receptor sites has been extended to assess the structure of agonists and antagonists bound to neuropeptide receptors and study their respective conformations, for example of dynorphin bound to the human κ-opioid receptor (O'Connor et al., 2015). These characterizations of neuropeptide and neuropeptide–receptor conformations should enable future advances in developing drugs to mimic or block neuropeptide binding.

Other spectroscopic methods have also been useful in the characterization of neuropeptides. Infrared (IR) spectroscopy has successfully been used to provide a quantitative estimate of secondary structural elements. When analyzed by IR spectroscopy, peptides demonstrate characteristic peaks for different folding patterns, including α-helices, β-sheets and turns, as has been characterized in human Peptide YY neuropeptide

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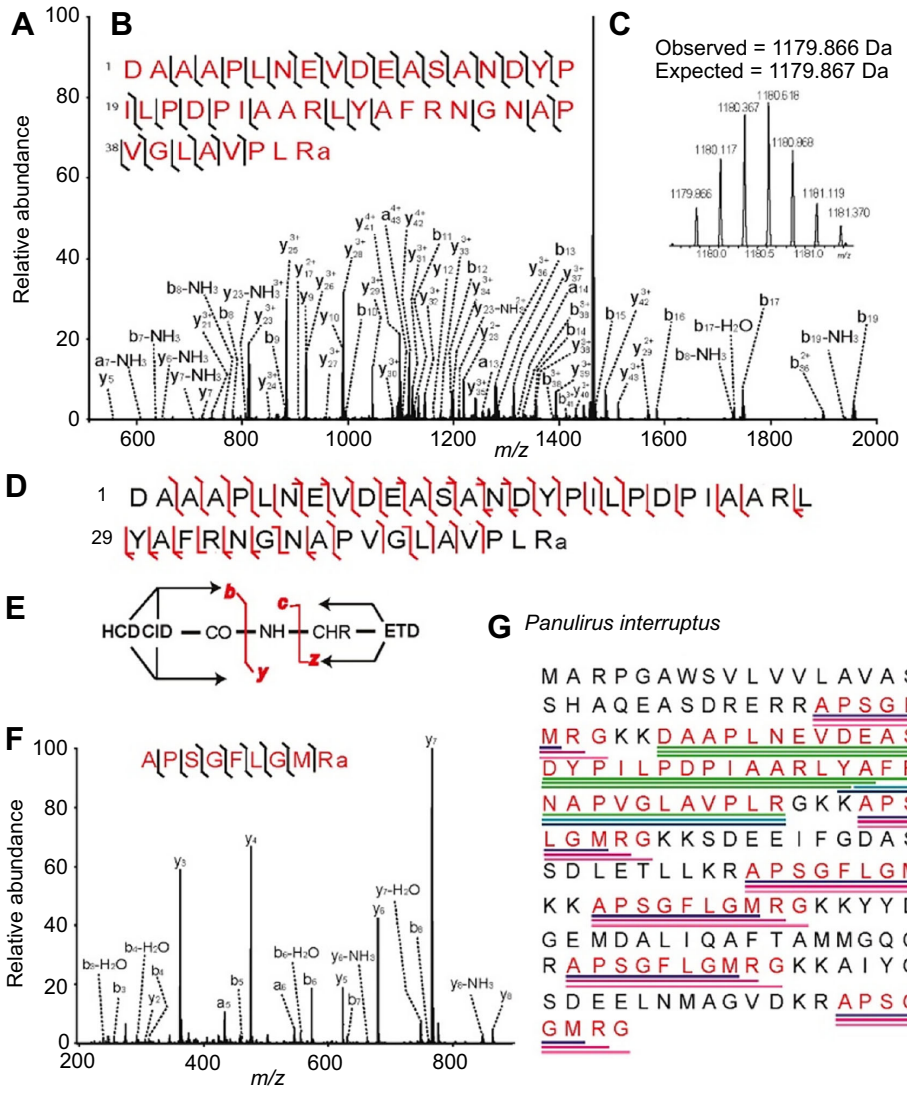

Fig. 2. Depiction of *de novo* sequencing of peptides by using MS. (A) High-resolution tandem MS with sequence-specific fragment ions annotated. (B) Sequence assignment based on fragmentations, in which mass differences between adjacent fragment ion peaks are matched to amino acids. The tick marks shown indicate locations of cleavage sites that led to the fragments detected in the spectrum. (C) Enlarged, high-resolution spectrum showing mass accuracy and isotopic distribution (necessary for measurements of differences between molecules with the same nominal mass). (D) Display of the sequence coverage. Each tick mark indicates the cleavage of the peptide yielding a fragment that has been detected in the MS spectrum. The more fragments that are detected, the greater the sequence coverage. (E) Representation of the different types of ions produced in tandem MS depending on fragmentation method used and bond cleavage sites. The cleavage sites are indicative of typical fragmentation patterns characteristic of the two common types of fragmentation methods. The b and y ions are produced during HCD and CID fragmentation, and c and z ions are produced during ETD fragmentation. (F) Tandem mass spectrum of another peptide (from Jonah crab *Cancer borealis* tachykinin-related peptide) with fragments indicated. (G) Comparison between peptides detected with MS (different colored lines indicate different detected peptides) and those predicted based on the precursor cDNA sequence of the spiny lobster *Panulirus interruptus* (highlighted in red). Adapted with permission from Ye et al. (2015). CID, collision-induced dissociation; ETD, electron transfer dissociation; HCD, higher-energy collisional dissociation.

(Hegefeld et al., 2011). Circular dichroism (CD) spectra have also shown utility in the rapid determination of secondary structure, and have provided evidence for the existence of α-helices in tachykinin-related peptides and β-sheets in melanocyte-stimulating hormone (MSH) peptides that increase with increasing charge state (Schneider et al., 2016). X-ray crystallography benefits from providing sub-angstrom resolution of key structural sites. The binding structures of neuropeptides with their receptors have been well characterized with X-ray crystallography, including studies of the receptor for neuropeptide S (Hassler et al., 2014) and human OX_2 receptor (Yin et al., 2014). Information from crystallography can provide useful details about neuropeptide structure that might lead to insights about function.

Methods for neuropeptide localization

Neuropeptide localization in species and/or tissue(s) enables mapping in neuronal subtypes relative to structural components of the cell, tissue or whole organisms, which can then be used to inform the function of a target neuropeptide and to direct functional biology experiments (Hoelters et al., 2016). Overall, the rapid increase in ‘omics’-derived neuropeptide sequence data has revolutionized our approach to the localization of neuropeptides and their signaling pathway components (Elphick and Mirabeau, 2014) and facilitated our ability to construct species- and neuropeptide-specific ‘connectomes’ (Shahidi et al., 2015). The application of localization techniques across species, and tissue and cell types, is fundamental to understanding the complexity of neuropeptidergic signaling and has trans-disciplinary importance (De Haes et al., 2015); indeed these techniques have been applied to cell cultures (2D, 3D or single cells) (Ahlf Wheatcraft et al., 2014; Janson et al., 2016; Zimmerman et al., 2011) and entire organisms (whole and sections) (Condro et al., 2016; Khatib-Shahidi et al., 2006). Neuropeptide localization in thick tissues, such as whole organisms or invertebrate brain tissue, can be achieved by using 3D mapping and ion density reconstruction of individual tissue sections to produce 3D representations of neuropeptide distributions (Chen et al., 2009). The advances in the tools and techniques described here have facilitated exploration of neural circuitry landscapes such that knowledge about neuropeptide localization and expression is accumulating rapidly.

Visualization or detection of neuropeptides at the cellular, tissue, whole organism or bio-fluid levels has been enabled by the application of radioimmunoassays (RIAs), immunohistochemistry (IHC), immunocytochemistry (ICC) and immunoelectron microscopy to the extent that these techniques have provided most of our former and current knowledge on the localization of

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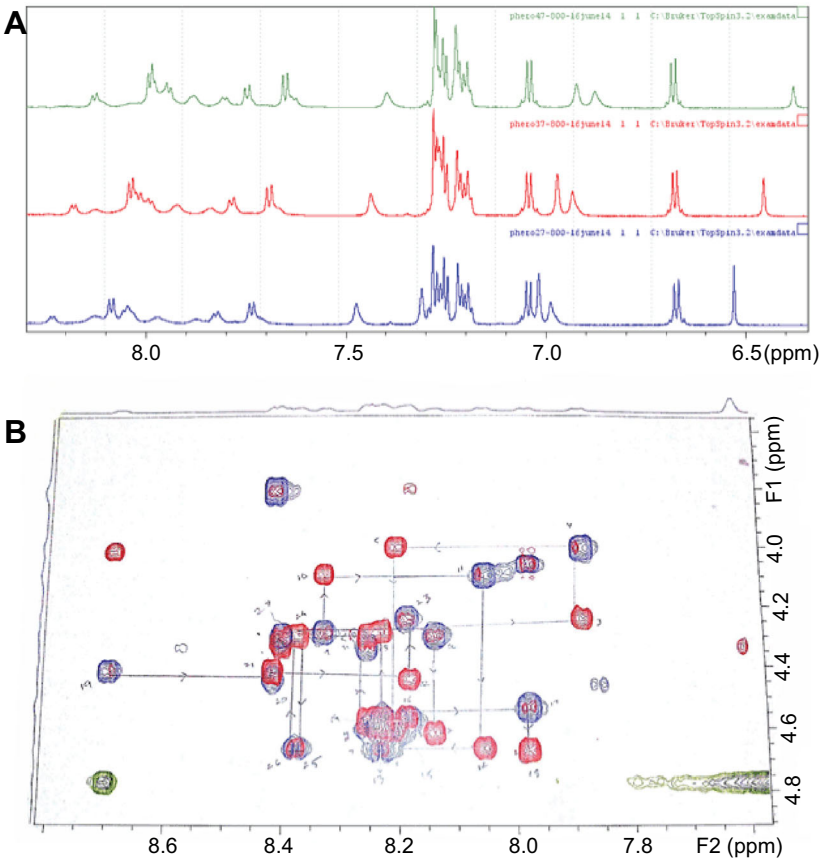

Fig. 3. NMR spectra of a peptide standard of the neuropeptide pheromonotropin, originally discovered in an extract from the head of *Mythimna* (*Pseudaletia*) *separata* (armyworm) larvae. (A) One-dimensional [^{1}H] NMR spectra collected at different temperatures, showing differences in chemical shift of NH protons in the peptide. The dependence of chemical shift on temperature is indicative of the degree of hydrogen bonding. Values below 3.00 ppb (chemical shift) per unit Kelvin indicate the presence of strong hydrogen bonds. As can be seen, the values for this peptide fall above that threshold, revealing that the protons are freely exposed to the solvent in this conformation. (B) Two-dimensional NMR spectra [total correlated spectroscopy (TOCSY) in blue, and rotating-frame Overhauser spectroscopy (ROESY) in red], showing a sequential assignment walk. The TOCSY spectrum provided information on NH-αH cross peaks, while cross peaks from the ROESY spectrum represent NH_i-$\alpha H_{(i-1)}$. Adapted with permission from Bhattacharya et al. (2015).

neuropeptides (Yalow and Berson, 1960). Compared with traditional histology-based approaches, these techniques enable enhanced specificity and sensitivity through the use of antibodies – for example, for the detection of the specific psychostimulant neuropeptide cocaine- and amphetamine-regulated transcript peptide (CART) (Singh et al., 2016). Antibodies can theoretically also be raised against virtually any peptide; however, many invertebrate neuropeptide genes encode more than one biologically active peptide that show high structural similarity to each other, leading to antibody cross-reactivity (Husson et al., 2009; McVeigh et al., 2009; Rowe and Elphick, 2012). Generation of N-terminally directed antisera, which can readily distinguish between peptides with highly similar C-terminal motifs, can help overcome cross reactivity issues (Atkinson et al., 2016). Another limiting factor is the number of peptides (and peptide signaling pathway components) that can be colocalized at the same time through traditional IHC and ICC approaches, which is in contrast to what is seen with MS-based peptidomics tools (see below) that enable the complete neuropeptide profile of the animal, tissue, organ or even a single cell to be deduced at any given time, readily enabling the identification of multiple colocalization events.

In situ hybridization (ISH) methods facilitate target-specific expression mapping of neuropeptide-encoding genes at the whole animal, tissue and single-cell level by determining the RNA localization. This involves hybridization of a single-stranded RNA oligoprobe and the complementary native mRNA sequence in the tissue or cell. The field of ISH and fluorescence ISH (FISH) has advanced significantly to enable the high-sensitivity detection of multi-target RNAs simultaneously in multiple species coupled with automated data collection and analysis systems (Levsky and Singer, 2003). There are several different approaches for detection of hybridized probes, including non-radioactive and radioisotope strategies. Regardless of the approach, careful consideration should be given to transcript abundance, where the detection of low-level or single-copy transcripts [e.g. of G-protein-coupled receptors (GPCRs)] can benefit from the use of target, signal or probe amplification techniques (Qian and Lloyd, 2003). Whilst one caveat of ISH is that the information it provides on RNA localization gives no definite indication of translated peptide distribution, it can relate valuable spatio-temporal information to gene activity when used in conjunction with ICC and IHC (Atkinson et al., 2016).

Reporter gene constructs encode proteins that function as site-specific gene expression markers when fused to the regulatory regions (promoters) of a gene of interest. They offer an alternative detection method to ISH that is useful for transcript detection in living cells and organisms. The method requires the promoter region of the neuropeptide gene and coding region of the reporter gene to be fused and inserted into the organism of choice for use as a reporter of gene expression. It is important that the reporter gene [which often encodes green fluorescent protein (GFP)] is non-native, assayed easily (e.g. by visual detection) and does not affect the normal physiology of the organism under study. The use of promoter::reporter gene constructs as localization tools is popular in model organisms such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, where transgenesis is readily achievable (Clynen et al., 2010; Husson et al., 2007; Kim and Li, 2004). A cautionary note should be given on the reliance on transcriptional reporters, however, as they do not always provide complete and reliable gene expression data in comparison to translational reporters that include important intron and 3′ untranslated region (UTR) regulatory elements (Turek et al., 2016).

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Techniques for localization of the complete neuropeptidome of an organism have seen progress with the use of MS since its recent development as a molecular imaging tool (Caprioli et al., 1997; OuYang et al., 2015b; Ye et al., 2012). Because no prior knowledge of the molecules is needed for analysis, theoretically hundreds or thousands of molecules can be imaged in one sample run. Among the various ionization sources available, MALDI has been the most prominent in imaging peptides and neuropeptides (Boggio et al., 2011; Ye et al., 2013), although success of detecting or identifying the neuropeptides is dictated by sample preparation and the detector coupled to the MALDI source. Time-of-flight-based instruments have a niche in analyzing larger neuropeptides with a fast speed, but the low resolution and sensitivity have motivated the development of alternative ionization techniques for larger neuropeptides, including matrix-assisted ionization in vacuum and laserspray ionization, which can be accomplished with commercial MALDI sources (Chen et al., 2016a; McEwen et al., 2010; Trimpin and Inutan, 2013). For example, Chen et al. were able to ionize an 18.7 kDa protein on a commercial MALDI-LTQ-Orbitrap XL, which is usually limited to molecules smaller than 4 kDa. It should be noted that some of the matrices required are more compatible with long imaging runs than others. Moving forward, refinements have recently been made in the optimization of sample preparation methods for MS imaging. For example, when sectioning tissue, only certain embedding materials are compatible with MS (Niehoff et al., 2014; Strohalm et al., 2011). Optimal cutting temperature embedding material is commonly used for the classical histology staining, but due to its polymer structure, it tends to suppress and mask the analyte signal, especially in the mass range of most neuropeptides. Another major problem is that, prior to any histology analysis, samples tend to be passed through the fixation process to help maintain tissue structure and deactivate any degradation processes, which limits neuropeptide MS analysis (Casadonte and Caprioli, 2011; Chaurand et al., 2008). Tissue fixation requires many washes, which may remove neuropeptides, and possibly a crosslinking step, which will make neuropeptides unavailable for extraction by the matrix and thus ionization. To complicate the situation further, the choice and application of the matrix is extremely important for the proper extraction of neuropeptides, and extensive effort has been devoted to developing better, more-effective methods (Gemperline et al., 2014; Guenther et al., 2011). For example, by utilizing electrospray deposition of the matrix α-cyano-4-hydroxycinnamic acid, researchers have imaged the FMRFamide neuropeptide family from a snail at a 5 µm spatial resolution, allowing confirmation of the localization found via IHC analysis (Márk et al., 2012). For more details, readers may consult recent reviews for MS imaging and its application to neuropeptides (Buchberger et al., 2015; Caprioli, 2015; Caprioli et al., 1997; Schmerberg and Li, 2013a).

In general, the reliance of mass matching for compound identification in MS imaging poses limitations in identification confidence. Owing to the low abundance of neuropeptides, performing tandem MS during imaging is often challenging. Therefore, accurate mass matching is the easiest way to identify a putative neuropeptide. The incorporation of high-quality tandem MS in a hybrid linear ion-trap–orbitrap instrument has provided improved *in situ* neuropeptide identification (Chen et al., 2010a; Römpp et al., 2010; Verhaert et al., 2010). The development of a spiral step method (Fig. 4), instead of the standard raster step, has allowed for further enhancement of chemical information by improving the depth of profiling and producing higher-quality images on Orbitrap-based instruments (OuYang et al., 2015a).

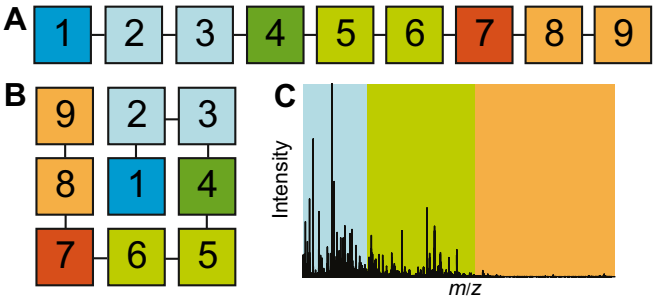

Fig. 4. In order to achieve better profiling depth during MS imaging of neuropeptides, a spiral step method has been developed. Instead of the classical raster step (A), a spiral square (B) is set up. In the example spiral, the square is broken into nine individual steps. The first square is an MS scan (dark blue), while the two following squares (light blue) are tandem MS scans. This repeats three times until all nine steps in the spiral are completed. Each square is a raster step of 50 µm, with the whole spiral being 150 µm. This system can be customized to balance MS and tandem MS scans. For example, step one could be an MS scan, while squares 2–9 could be tandem MS scans if the user desires. Furthermore, this method can be targeted or used with data-dependent acquisition (DDA). For DDA experiments, the highest intensity peaks are chosen for tandem MS analysis. Since neuropeptides tend to be in low abundance compared to lipids and have a wide mass range, we can segregate the spiral step method into multiple mass ranges (e.g. three) to improve sampling of neuropeptides (C). The distinct *m/z* ranges are shown in the three different colors in the spectrum (OuYang et al., 2015a).

Another technique, IMS, has the potential to be used before detection and to remove interfering molecules and thus increase the image quality (Sturm et al., 2014). Overall, the unparalleled chemical information and multiplexing capacity offered by MS imaging technology provides an attractive tool for high-throughput mapping the localization of neuropeptides, although the inherent limitations of laser beam size and matrix crystal size of the MSI technique prevent it from having the same spatial resolution offered by IHC/ICC approaches. Further technology development is needed to improve these aspects and sensitivity to allow MS imaging to become a central tool for neuropeptide localization in the nervous system.

Recent advances in bioimaging and microscopy tools in parallel with upgrades in computer processing and digital storage capabilities have significantly enhanced the ability to capture and describe the neuroanatomy of invertebrates. Traditionally, light microscopy has been used in invertebrate neuroscience research to map the coarse architecture of the nervous system, with electron microscopy being employed for fine ultrastructural analysis (Schmidt-Rhaesa et al., 2016). More recently, confocal and multiphoton microscopy have facilitated the generation of high-resolution 2D and 3D images of both thicker whole-mount and live specimens (Bixel et al., 2015). Laser microdissection tools provide an alternative to labor-intensive antibody-based experiments by enabling the post-capture profiling of neuropeptides (e.g. via RNAseq) in specific neurons or in tissues embedded in heterogeneous samples (Fricker, 2012). Advances in image-analysis software programs make the comparative quantification of neuropeptides in the nervous system more streamlined, and facilitate the integration of optical imaging technologies into the functional genomics ‘toolbox’ (Atkinson et al., 2013; Robichaud et al., 2013). Additionally, integrating multimodal imaging studies through MALDI-MS imaging and microscopy-based imaging could provide enhanced spatial and chemical information for neuropeptide localization.

Web-based databanks for curating neuropeptide data in invertebrates are a much-needed resource that will greatly

facilitate invertebrate neuropeptide research and enable inter- and cross-phyla comparative analyses, in addition to providing a 'go to' repository for researchers. These types of resources are currently available for a number of invertebrate phyla and provide a range of data types in user-friendly formats (Yeoh et al., 2017); the database NeuroPep collates pan-phylum data and enables comparisons of neuropeptide structure, expression and function (Wang et al., 2015). Furthermore, the availability of species-specific anatomical maps of the nervous system is essential for the precise and comparable morphological description of peptidergic neurons in invertebrates. These data are currently available for only a few invertebrate species (Menzel, 2012), including key model organisms [for example, *C. elegans*: Wormatlas (Altun et al., 2002-2018); *Drosophila melanogaster*: Neurokernal, Virtual Fly Brain (Armstrong et al., 1995; Chiang et al., 2011; Givon and Lazar, 2016); zebrafish: Z-brain (Randlett et al., 2015); and *Macrostomum lignano* (Morris et al., 2007)]; however, they are all at different stages of completion and vary in terms of their resolution, presentation and data source. These data are extremely valuable to our understanding of invertebrate nervous system structure and function and will inform functional biology. Efforts to generate similar maps for other species of interest are under way, but significant attention and support should be directed to the curation and maintenance of these resources, as there are many online databases that are no longer active owing to the termination of funding (Katz et al., 2010).

Assessing the function of neuropeptides

While the identification of neuropeptides is important, understanding their role in the nervous system is key to finding further applications. Understanding function is extremely difficult, as neuropeptides can have completely different functions within different tissues. Furthermore, even slightly different neuropeptide isoforms from the same family can have drastically different effects. Interestingly, even with the development of new, technologically advanced alternatives, older, well-vetted methods are still present in the literature either as a method of analysis or to confirm the observed results (Bilgic et al., 2016; Liu et al., 2016a). Function can be explored at many levels, ranging from the macro (e.g. behavioral) to the molecular scale (e.g. signaling pathways). Localization can also aid elucidation of neuropeptide function, as the tissue(s) a peptide is localized in may provide key clues about its role in the organism (Bruzzone et al., 2006). A variety of functional biology tools and techniques can be employed to determine the function of a neuropeptide, including those applied in either *in vivo* or *in vitro* settings. Two major approaches for functional analysis will be discussed below: altering the neuropeptide content and measuring neuropeptide levels.

Altering neuropeptide content

The most commonly performed and observed *in vivo* studies involve assessing behavioral and/or physical changes arising from the introduction of a neuropeptide into an organism or by using reverse genetics (RNA interference) to downregulate a specific neuropeptide (e.g. using siRNAs) (Bayerl et al., 2016; Lin et al., 2016). These approaches can provide a range of information from a general understanding of the physiological function of a neuropeptide to judging whether neuropeptides are therapeutically active (Wickström et al., 2004; Zhang et al., 2016). Neuropeptides, their antagonists and siRNAs can be delivered to an organism in several different ways, including by injection (Javadian et al., 2016; Narváez et al., 2016), incubation in media containing molecules (Chen et al., 2016b; Kasica et al., 2016) and even microdialysis (Torregrossa and Kalivas, 2008). siRNAs, RNA molecules that interfere with an expression of a gene, may require more sophisticated methods of delivery (e.g. transfection) or can also be injected for the induction of gene-silencing and thus knockdown of the neuropeptide (Flores-Burgess et al., 2017; Yang et al., 2017). Beyond introducing a neuropeptide or a neuropeptide antagonist, neuropeptide production can be altered at the genetic level in organisms, such as mice, through the production of knockout or transgenic animals, or through targeted genome editing approaches (e.g. the CRISPR/Cas system) (Hay et al., 2017; Shao et al., 2016; Van Sinay et al., 2017). CRISPR/Cas has gained a lot of popularity for its speed, ease of use and efficiency compared to other methods used to knockout genes or create transgenic animals (Hay et al., 2017). It should be noted that this technology is new and can be expensive to implement on a large scale. Furthermore, its application is not possible in non-model organisms. In all the cases described above, careful planning is required to determine the most appropriate and applicable technique to alter neuropeptide levels in an organism.

After alteration of the neuropeptide content, several behavioral observations or tests can be performed to assess change. Examples of behavior tests for animals (e.g. Wistar rats) are open-field-based or maze-based tests (Bahaaddini et al., 2016; Chrousos and Gold, 1992), and these tests are applicable to numerous species, including invertebrates such as planarians and *C. elegans* (Hagstrom et al., 2016; Qin and Wheeler, 2006). While these tests are easily performed and are normally the starting point for functional studies (Chu et al., 2016), behavioral studies are based on observations, meaning that data misinterpretation or choice of test to monitor changes can produce misleading data. Thus, care should be taken on choosing the most appropriate tests, methods or animal models to assess behavioral changes attributable to application of neuropeptides.

As neurons transmit signals through electrical currents, another facet of function to consider is electrophysiology. By selective or global activation, researchers are able to understand synaptic mechanisms by which neurons communicate and modulate their electrical activities (Fig. 5) (Kuksis and Ferguson, 2017). These

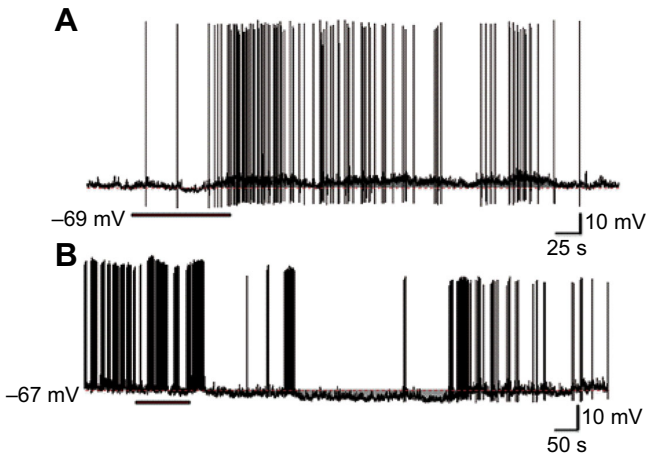

Fig. 5. A graphical representation of whole-cell patch clamp electrophysiology readings. In this image, subfornical organ neurons from rat brains are being exposed to 10 nmol l^{-1} nesfatin-1, an anorexigenic neuropeptide, at the time frame indicated by the line under the graph. When exposed, neurons can either become slightly depolarized, which is associated with an increase in firing frequency (A) or slightly hyperpolarized, which is associated with a decrease in firing frequency (B). Adapted with permission from Kuksis and Ferguson (2017).

readings can be performed in a few ways depending on the goal of the study, including intracellular versus extracellular electrophysiological recording (Matthews and Lee, 1991), whole-cell versus whole network (Beenhakker et al., 2004; Kuksis and Ferguson, 2014; Qiu et al., 2016; Zhao et al., 2016) or *in vitro* versus *in vivo* (Beenhakker et al., 2004; Li et al., 2016; Marder and Bucher, 2007; Nusbaum et al., 2017). In terms of neurological studies, *in vitro* whole-cell recordings are the most common, although *in vivo* live-animal recordings, which are inherently more difficult, are becoming more refined (Scanziani and Häusser, 2009). Crustacean model systems have been used heavily for electrophysiological studies (Daur et al., 2016; Dickinson et al., 2016; Otopalik et al., 2017). For example, the effects of neuromodulators on the same neuronal circuit was explored for the Jonah crab gastric mill motor pattern, which was interestingly explained by using a mathematical model (Kintos et al., 2016). In general, to better understand neuronal modulation at the single-neuron and network level, crustaceans provide an excellent model to derive detailed knowledge about synaptic mechanism and neuronal connection owing to their possession of a much simplified system compared with the mammalian system (Marder et al., 2017). Interestingly, the coupling of electrophysiological probes for simultaneous monitoring of other chemicals has been incorporated recently. This could include selective applications (e.g. oxygen, glucose) (Li et al., 2016) or could be more global, such as microdialysis, which would allow for direct dosing of neuropeptides (Szabo et al., 2011). Notably, some researchers believe that electrophysiology, while never replaceable, might be overshadowed or combined with other optical imaging techniques that allow localization of the neurological signals (Scanziani and Häusser, 2009). One can postulate that combining the temporal resolution of classical electrophysiology and spatial resolution of optical imaging could lead to significant discoveries in neuroscience.

Measuring neuropeptide levels

Physiological changes related to neuropeptide actions are most commonly studied *in vivo* by performing quantitative analyses on the whole organism. It should be noted that this can be the first step in many cases to understanding the function of a peptide, as it might not be known that a neuropeptide is involved in a process until it is administered or a condition is applied (e.g. a change in environment) (Zhang et al., 2016). After the organism has been exposed to the neuropeptide or changed condition, it can be euthanized and the tissues of interest collected for quantitative comparison (Abels et al., 2016). Alternatively, the tissue can be removed from the animal and incubated before analysis (Peng et al., 2016). Classically, this has been performed for individual proteins by western blotting, which is still widely reported in the current literature (Bayerl et al., 2016; Liu et al., 2016a). Owing to the typically small size of neuropeptides, western blotting is normally used to assess other, related protein changes or expression of neuropeptide receptors (Bayerl et al., 2016; Liu et al., 2016b). Other complementary examples of a targeted technique are enzyme-linked immunosorbent assay (ELISA) (Bilgic et al., 2016; Javadian et al., 2016) and use of radioactively labeled ligand and a γ-counter (Dhuria et al., 2016). While these methods are excellent if one has a target of interest, a non-biased global view of the dynamic changes of all the neuropeptides is often needed to understand fully the role of neuropeptides and their possible function at the system level.

With the advancement of technology, MS is becoming a useful addition to functional studies as it is able to reveal changes in neuropeptide levels that might correlate with function. This technique is especially attractive for analysis of organisms without a sequenced genome, as no prior knowledge of the molecule, such as the metabolite, protein or peptide, is needed. To highlight some key areas of success, crustacean neuropeptide research has benefited from MS, allowing researchers to quantify several neuropeptide changes arising from stress caused by changes in salinity or temperature (Chen et al., 2014; Zhang et al., 2015). Microdialysis of neuropeptides has also been coupled to MS, and several reviews highlight considerations for coupling these two techniques (OuYang et al., 2015b; Schmerberg and Li, 2013a). Multiplexed quantification beyond duplex has been implemented in proteomics (Frost et al., 2015; Wang et al., 2010; Xiang et al., 2010). In this technique, by using different combinations of stable isotopes (i.e. ^{13}C, ^{15}N, ^{2}H and ^{18}O), samples are differentially labeled prior to being mixed and analyzed together during the MS. It is expected that multiplexing will be applied more commonly in neuropeptide analysis of multiple samples (Bark et al., 2009; Che and Fricker, 2005). Although the use of MS is attractive, the depth at which it can profile depends upon many instrument characteristics, such as analysis time, resolution and mass range. Using an analyte target list can increase neuropeptidomic coverage, although sensitivity and interfering species can introduce difficulties. Owing to the natural complexity of biological samples, the coupling of separations to MS has not only improved detection but also enabled accurate quantification. This coupling includes capillary electrophoresis (CE), LC or IMS (Buchberger et al., 2015; Sturm et al., 2014; Zhong et al., 2014) before MS detection. By reductive dimethylation of comparative samples before CE separation, it has been shown that neuropeptides can be separated and quantified accurately, allowing for more-in-depth profiling (Warkiani et al., 2016; Zhang et al., 2012). Furthermore, new instrument methods, such as analysis at the MS3 level, have helped facilitate accurate quantification (Ting et al., 2011). It should be noted that, from these data, individual peptides can be selected for further analysis and validation by the above, targeted, methods. Finally, MS data are inherently more complicated, and the use and development of appropriate software to predict, identify or quantify is challenging, but necessary, for neuropeptidomics to continue progressing (Fälth et al., 2006; Hook and Bandeira, 2015; Ma et al., 2003).

Upon understanding the peptide changes, gene analysis can be conducted to help provide information about the global impact on the plasticity of the system. Although the specific neuropeptide, its propeptide and its pre-propeptide cannot be differentiated from each other at the transcript level, global gene analysis is most easily achieved by measuring the mRNA changes by using a quantitative real-time PCR (qPCR) technique (Caers et al., 2016; Peng et al., 2016). This approach differs from western blotting and ELISAs, the latter of which measures the translated peptide but does not require antibodies. By using qPCR, it has been shown that dosing of amphetamine not only affects rat food intake but also affects hypothalamic mRNA levels of neuropeptide Y (Chu et al., 2016). While more mRNA usually means enhanced gene expression, protein levels do not always correlate with the mRNA data, and the use of an orthogonal method (see above) should be performed to verify any conclusions. This is true for all of the methods above, as all of them have different advantages and disadvantages.

The use of modified peptide-gated channels as a tool to study neuroscience

The effect of a neuropeptide is ultimately determined by its receptor. While GPCRs mediate slow and more-modulatory neurotransmission by changing the membrane potential, ion

channel receptors, by contrast, mediate fast and transient neurotransmission, rapidly depolarizing or hyperpolarizing the postsynaptic membrane. It has been common knowledge for decades that neuropeptides mainly bind to and activate GPCRs, rather than ion channel receptors. There are now a few exceptions to this rule: ion channels directly activated by neuropeptides have been cloned and functionally characterized from different snails (molluscs) and the freshwater polyp *Hydra* (Cnidaria), unambiguously demonstrating the existence of ion channel receptors for neuropeptides in different animal phyla (Fig. 6). Moreover, genomic data have revealed the presence of related channels in other phyla, and electrophysiological data suggest the existence of a peptide-gated Cl^- channel in the nematode *Ascaris suum* (Holden-Dye et al., 1997; Purcell et al., 2002a,b). Thus, we speculate that the distribution of peptide-gated channels is at present vastly underestimated and that they might mediate some of the physiological functions of neuropeptides in several animals; maybe even in humans, although this is, at present, thought to be unlikely. Here, we will briefly describe the discovery of the known peptide-gated channels, introduce their properties and then focus on how they might be developed into tools for neuroscience.

The first observations of a peptide-gated ion channel were made by Cottrell and co-workers, who showed that the cerebral C2 neuron of the snail *Helix aspersa* is rapidly excited by the neuropeptide FMRFamide (Cottrell et al., 1990; Green et al., 1994). Peptides related to FMRFamide, RFamide neuropeptides, are found in many animals. The excitation was fast and also observed in outside-out patches containing 5′-O-(2-thiophosphate), which blocks G-protein-coupled responses, in the patch pipette (Green et al., 1994). These results strongly suggested that FMRFamide directly activated ion channels in these neurons. The currents were Na^+-selective and sensitive to the diuretic amiloride (Green et al., 1994). These biophysical and pharmacological properties are reminiscent of the epithelial Na^+ channel (ENaC) from vertebrates, and, in 1995, by means of homology to ENaC, the FMRFamide-gated Na^+ channel (FaNaC) was cloned from *H. aspersa* (Lingueglia et al., 1995) – the first peptide-gated channel. A single FaNaC subunit is sufficient to produce functional channels with properties similar to the native channel in C2 neurons: they are Na^+ selective and sensitive to amiloride (EC_{50}=0.6 µmol l^{-1}; Table 2) (Lingueglia et al., 1995). Although it was reported that FaNaC is a tetramer (Coscoy et al., 1998), there is now compelling evidence from crystallization of closely related acid-sensing ion channels (ASICs) (Jasti et al., 2007), as well as from single-molecule imaging (Bartoi et al., 2014; Chen et al., 2015), that channels of the degenerin (DEG)/ENaC gene family have a trimeric stoichiometry (Fig. 6A). In addition, species orthologs of FaNaC have been cloned from three other molluscs, including *Aplysia* (Furukawa et al., 2006; Jeziorski et al., 2000; Perry et al., 2001), but so far no additional subunits have been cloned. Thus, although it cannot be ruled out formally that the native channel contains other subunits, it is likely that FaNaC functions as a homotrimer. Table 2 provides an overview of the properties of known peptide-gated channels.

In 2007, by means of homology to ENaC and FaNaC, four related subunits were cloned from the freshwater polyp *Hydra* (Golubovic et al., 2007), which belongs to the ancient phylum Cnidaria. It was found that two of them, when co-expressed in a heterologous expression system, formed an ion channel that was directly activated by two neuropeptides (Golubovic et al., 2007), which had been previously isolated from the *Hydra* nervous system using a RIA (Moosler et al., 1996). Like FaNaC, the channel also conducts Na^+ and therefore was named the Hydra Na^+ channel (HyNaC). These two neuropeptides, Hydra-RFamides I and II, share a C-terminal RFamide group with FMRFamide, the ligand of FaNaC. HyNaC is not the species ortholog of FaNaC; however, as it is more closely related to mammalian ASICs than to FaNaC or ENaC (Golubovic et al., 2007), it is likely that peptide-gated channels are ancient and evolved before the cnidarian–bilaterian split. Three years after the identification of these neuropeptides, another HyNaC subunit was cloned that assembles with the two previously cloned subunits, suggesting that the native channel is a heterotrimer containing three different subunits (Dürrnagel et al., 2010). In contrast to FaNaC,

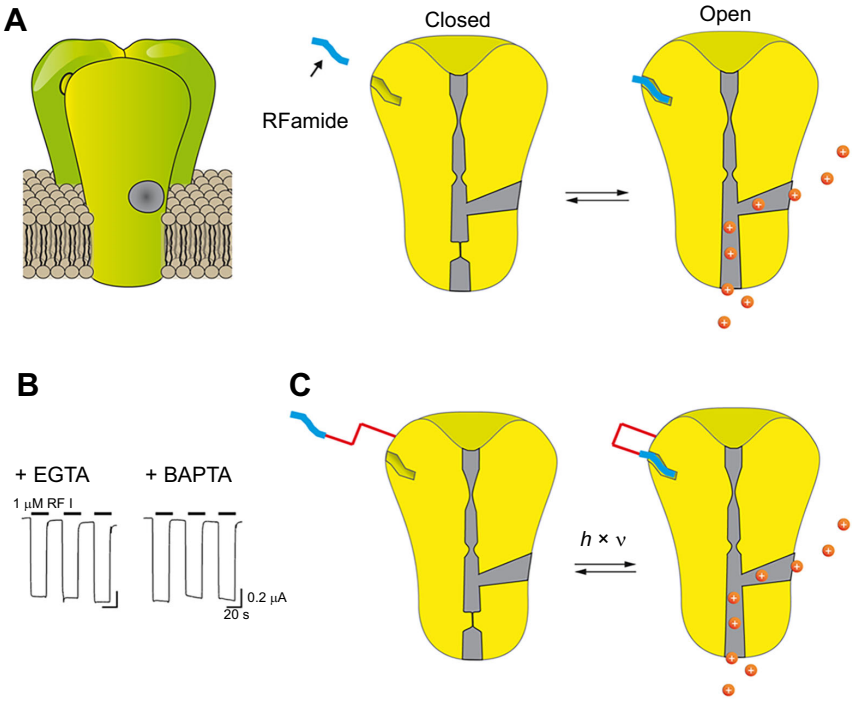

Fig. 6. Properties of peptide-gated HyNaCs. (A) Left, cartoon illustrating the three-dimensional structure of a channel. The ligand-binding site is unknown and is drawn here at the interface of two subunits for illustration. Right, HyNaCs can be either open or closed. The equilibrium between these two conformations is shifted by binding of a RFamide peptide (blue) to the extracellular domain. (B) HyNaCs can be repeatedly activated by their ligand, *Hydra* RFamide I (RF I), and do not desensitize. The inward current is carried by Na^+ and Ca^{2+} (orange circles) Used with permission from Dürrnagel et al., 2012. (C) Cartoon illustrating how a peptide covalently linked to the channel could be moved into and out of its binding site by application of light via a photoisomerizable linker (a 'light-switch', red).

Table 2. Properties of peptide-gated ion channels

Channel	Gene family	Stoichiometry	Ligand	Ligand affinity	Kinetics	Ion selectivity	Pharmacology
FaNaC	DEG/ENaC	Homo-trimer	FMRF-NH_2	2–70 µmol l^{-1}[a,b]	Partially desensitizing (τ ~1 min)[c]	Na^+-selective P_{Na}/P_K>10[a]	EC_{50}(amil)=0.6 µmol l^{-1}[a]
HyNaCs	DEG/ENaC	Hetero-trimer[e,f]	pQWLGGRF-NH_2 pQWFNGRF-NH_2	0.04–>30 µmol l^{-1}[e,f]	Non-desensitizing[f,g]	Cation-unselective P_{Na}/P_K=3[g] P_{Ca}/P_{Na}=4[g]	EC_{50}(amil)=100 µmol l^{-1}[e] EC_{50}(dimi)=0.05–5 µmol l^{-1}[f]
Ascaris suum channel	Unknown	Unknown	KPNFLRF-NH_2 (or similar)	0.001–0.1 µmol l^{-1}[h,i]	Non-desensitizing[h,i]	Cl^--selective[h,i]	

Amil, amiloride; dimi, diminazene.
[a]Lingueglia et al., 1995, [b]Jeziorski et al., 2000, [c]Kodani and Furukawa, 2010, [d]Golubovic et al., 2007, [e]Dürrnagel et al., 2010, [f]Assmann et al., 2014, [g]Dürrnagel et al., 2012, [h]Holden-Dye et al., 1997, [i]Purcell et al., 2002a.

HyNaC is an unselective cation channel with a high Ca^{2+} permeability (Dürrnagel et al., 2012) (Table 2). Soon after, all 12 DEG/ENaCs of *Hydra* were cloned, and it was shown that *Hydra* likely contains at least six different functional HyNaCs (Assmann et al., 2014). All are heterotrimers consisting of three different subunits activated by Hydra-RFamides I and II, and all are unselective cation channels (Assmann et al., 2014) (Table 2). It is not clear why *Hydra* evolved such a variety of peptide-gated channels with similar properties, but differential targeting and ligand affinity are two possibilities.

ISH revealed that two of the six HyNaCs are most likely expressed in epitheliomuscular cells at the oral side of the tentacle base, two at the aboral side and two in the foot region (Assmann et al., 2014). Application of amiloride or diminazene, two inhibitors of HyNaCs (Table 2), delayed the feeding reaction of living *Hydra* (Assmann et al., 2014; Dürrnagel et al., 2010), which is characterized by a bending of the tentacles. Collectively, these results suggest that the *Hydra* RFamide peptides are released at neuromuscular junctions and that HyNaCs contribute to fast neuromuscular transmission (Gründer and Assmann, 2015).

Usually, ligand-gated ion channels desensitize in the continued presence of the ligand. This feature, together with rapid re-uptake or hydrolysis of small-molecule transmitters, makes transmission with ligand-gated channels transient. HyNaCs, by contrast, could also mediate longer-lasting depolarization of the postsynaptic membrane – they do not desensitize (Dürrnagel et al., 2012) (Fig. 6B), and there is no known rapid re-uptake mechanism for their ligand. In combination with their high Ca^{2+} permeability, these features could endow HyNaC-expressing cells with an efficient entry path for extracellular Ca^{2+}, which could be important for muscle contraction (Gründer and Assmann, 2015).

DEG/ENaCs with high levels of sequence similarity to either FaNaC or HyNaCs are present in several genomes, for example in that of *Nematostella vectensis*, a cnidarian that belongs to the subphylum Anthozoa that is not closely related to Hydrozoans, and in that of the placozoan *Trichoplax adhaerens* (Gründer and Assmann, 2015). As *T. adhaerens* does not contain a nervous system, the presence of putative peptide-gated channels in this organism suggests that the channel–peptide-ligand system predated the emergence of nervous systems and might have a role for example in paracrine signaling. Molecular cloning and functional analysis of these channels will improve our understanding of the physiological function of peptide-gated channels.

In addition to their importance in understanding neurotransmission in different organisms, peptide-gated ion channels might also be modified into interesting tools for neuroscience. For example, FaNaC has been used as a reporter of neuropeptide release that achieves high temporal resolution (Whim and Moss, 2001). FMRFa has been used to tag a neuropeptide prohormone, and FaNaC has acted as a reporter to monitor release of FMRFa and thereby also of the tagged neuropeptide (Whim and Moss, 2001).

In another example, it has been shown that heterologous expression of FaNaC in mammalian hippocampal neurons provides a means to depolarize the neurons and induce bursts of action potentials upon focal application of FMRFa (Schanuel et al., 2008). FaNaC has a somato-dendritic localization and is absent from axons (Schanuel et al., 2008). As FMRFa is not present in the mammalian nervous system, and endogenous RFamides apparently do not activate FaNaC (Schanuel et al., 2008), it is in principle possible to activate specific subsets of neurons selectively in intact nervous tissue. Transgenic expression of FaNaC under the control of specific promoters would enable driving of its expression only in specific subsets of neurons in living animals. Moreover, the possibility to 'cage' FMRFa chemically with a photolabile protecting group allows its release within milliseconds upon exposure to both single- and two-photon light sources (Janett et al., 2015) to rapidly excite cells expressing FaNaC. As HyNaCs are obligate heteromers, their heterologous expression in neurons is more difficult, but would allow expression of a foreign ion channel with high Ca^{2+} permeability. The cloning of further peptide-gated channels, such as the Cl^- channel from *A. suum*, will further increase the toolbox of peptide-gated channels.

A better understanding of the molecular binding site of peptide ligands on their ion channel receptors could also allow the future design of small molecules that gate the channels independently of peptides. This might allow peptide-gated channels to be employed, much like some GPCRs, as 'designer' receptors exclusively activated by designer drugs (DREADDs) (Roth, 2016).

The identification of the peptide-binding site might also allow the covalent attachment of FMRFa (or other peptides) close to its binding site via a photoisomerizable molecule (a 'photoswitch') such that light would move the peptide in and out of its binding site to open and close the channel (Berlin and Isacoff, 2017; Kramer et al., 2009) (Fig. 6C). Azobenzenes have been successfully used as such photoswitches, as they undergo fast trans-to-cis isomerization, much like retinal, upon illumination with near-UV light (Berlin and Isacoff, 2017). They can be coupled via maleimides to single cysteine residues engineered into the primary sequence of a channel. High-resolution structures are not only useful for the identification of the peptide-binding site but also a pre-requisite for the identification of suitable attachment sites of peptide ligands close to the binding site. As chicken ASIC1, a close homolog of HyNaCs, has been crystallized (Jasti et al., 2007), appropriate homology models of the HyNaC structure, and perhaps also of the FaNaC structure, could feasibly be constructed. Such photo-sensitive channels would allow experimenters to control the membrane potential of a neuron by light instead of a peptide ligand. Examples

that such a synthetic optogenetics approach (Berlin and Isacoff, 2017) is feasible have been provided, among others, for ionotropic glutamate and $GABA_A$ receptors (Lin et al., 2015; Volgraf et al., 2006). Clearly, peptide-gated ion channels have great potential to serve as useful tools for neuroscience.

Concluding remarks

It is clear that neuropeptide research has benefited tremendously from the substantial advancements of technology for neuropeptide structural elucidation, localization mapping and functional understanding, although any single technique itself still does not provide us with all the answers we seek. A particularly promising technique is MS imaging with tandem MS, but sensitivity issues might be limiting when single-cell resolution is needed. In general, the development of the MS-based neuropeptidomics technique has proven to be the most influential technique for analyzing neuropeptides in a high-throughput and global manner, but other, often classical, methods provide validation and confirmation of all results generated by MS. Owing to its ability for global analysis, MS will likely be a central tool for all future neuropeptide studies, especially with the continued development of new methodology and technology. Furthermore, another area that has proven to be extremely influential has been computational prediction and processing. Without sophisticated bioinformatics tools, not only would the identification of novel neuropeptides be slow, but MS datasets, which are naturally large and complex, would be extremely difficult to process and interpret. At the moment, the pace of investigating the neuropeptidome will continue to be set by the development of both of these areas, although new techniques that are complementary or capable of providing structure, function and localization information are welcome additions to the study of neuropeptides. In addition, peptide-gated ion channels might be modified into promising new tools for neuroscience. Finally, the integration of multiple bioanalytical techniques and molecular neuropharmacological tools will drive the field of neuropeptide research towards new frontiers.

Competing interests

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